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NBS1 Localizes to γ-H2AX Foci through Interaction with the FHA/BRCT Domain

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Summary

DNA double-strand breaks represent the most potentially serious damage to a genome; hence, many repair proteins are recruited to nuclear damage sites by as yet poorly characterized sensor mechanisms. Here, we show that NBS1, the gene product defective in Nijmegen breakage syndrome (NBS) [1–3], physically interacts with histone, rather than damaged DNA, by direct binding to γ-H2AX. We also demonstrate that NBS1 binding can occur in the absence of interaction with hMRE11 or BRCA1. Furthermore, this NBS1 physical interaction was reduced when anti-γ-H2AX antibody was introduced into normal cells and was also delayed in AT cells, which lack the kinase activity for phosphorylation of H2AX. NBS1 has no DNA binding region but carries a combination of the fork-head associated (FHA) and the BRCA1 C-terminal domains (BRCT) [4]. We show that the FHA/BRCT domain of NBS1 is essential for this physical interaction, since NBS1 lacking this domain failed to bind to γ-H2AX in cells, and a recombinant FHA/BRCT domain alone can bind to recombinant γ-H2AX. Consequently, the FHA/BRCT domain is likely to have a crucial role for both binding to histone and for relocalization of hMRE11/hRAD50 nuclease complex to the vicinity of DNA damage.

Results and Discussion

Genomic integrity is maintained by dynamic responses to DNA damage, in which several factors for chromatin remodeling and DNA repair are involved. Histone H2AX is phosphorylated and forms discrete foci immediately (within 5 min) after irradiation [5]; hence, it may represent an earlier signaling response than formation of the NBS1/hMRE11/hRAD50 (N/M/R) complex on damaged sites. To investigate whether H2AX is phosphorylated in the absence of NBS1, phosphorylation of H2AX (γ-H2AX) was assayed in a normal lymphoblastoid cell line (GM2184c) and in NBS1-mutated NBS cells (94p548). Phosphorylation was detected in the irradiated NBS cells and in normal cells (Figure 1A). Consistent with the phosphorylation, γ-H2AX foci were observed 30 min after 2 Gy of irradiation in both NBS cells and in NBS1-complemented cells (NBS1cDNA-transfected NBS clones), while these foci were gradually decreased 5 hr later (Figure 1B).

Although γ-H2AX foci formation occurred first, most γ-H2AX foci in HeLa cells colocalized with foci of N/M/R complex within 2 hr of irradiation, as detected by anti-hMRE11 antibody (Figure 2A). In the absence of NBS1, hMRE11 protein was confined to the cytoplasm and did not form nuclear foci, while γ-H2AX foci were detected (Figure 2A). Subsequently, we examined potential protein interactions by using immunoprecipitation assays. A small amount of NBS1 was detected in immunoprecipitates by using anti-γ-H2AX antibody in nonirradiated normal cells, possibly during S phase [6], and this amount was significantly increased after irradiation with 10 Gy (Figure 2B). Similarly, γ-H2AX coimmunoprecipitated with anti-NBS1 antibody (Figure 2C). hMRE11 was also present in this NBS1/γ-H2AX immuno-complex, while the amount of hMRE11 did not parallel that of NBS1, probably due to the direct binding of hMRE11 to DNA. However, when NBS cells were irradiated, hMRE11 was not detected in immunoprecipitates with anti-γ-H2AX antibody (Figure 2B), and this indicates that hMRE11/hRAD50 cannot interact with γ-H2AX in the absence of NBS1 and that N/M/R complex interactions with γ-H2AX are most likely mediated by binding of NBS1 to γ-H2AX.

These experiments did not allow us to exclude the possibility of unknown intermediate cofactor(s), which might bridge NBS1 and γ-H2AX. Therefore, H2AX-N/M/R interactions were investigated by in vitro experiments with recombinant proteins. When a mixture of recombinant H2AX and recombinant full-length NBS1 was immunoprecipitated with anti-H2A antibody (recognizes both H2AX and γ-H2AX), the precipitate did not contain recombinant NBS1 (Figure 3A). However, after phosphorylation of recombinant H2AX by ATM kinase, the immunoprecipitate using anti-H2A antibody contained recombinant NBS1 and γ-H2AX. This demonstrates that NBS1 binds directly to γ-H2AX and that this binding is specific for the phosphorylated form of H2AX protein. Moreover, recombinant γ-H2AX showed no binding to a recombinant C-terminal NBS-His-tagged protein but did bind to a FHA/BRCT-GST-tagged protein (Figures 3A and 3B), demonstrating the requirement of FHA/BRCT domain for this direct binding.

NBS1 has two conserved domains; the ten C-terminal

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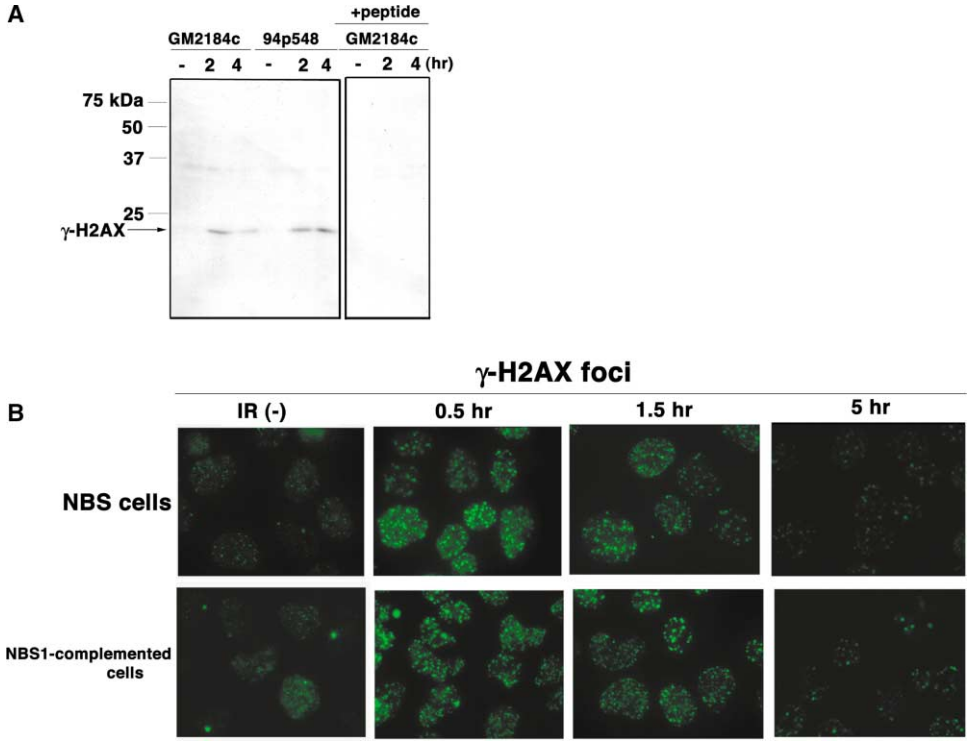

Figure 1. Histone H2AX Was Phosphorylated and Formed Foci in NBS Cells

(A) Normal (GM2184c) and NBS (94p548) lymphoblastoid cells were irradiated with 10 Gy of ^{60}Co γ rays, and phosphorylations of H2AX were measured at indicated times by a Western blot with anti-γ-H2AX antibody. The specificity of anti-γ-H2AX antibody was confirmed by the disappearance of the signal after adding γ-H2AX phospho-peptide to the primary antibody (+peptide).

(B) NBS fibroblast cell line (GM07166VA7) and NBS-complemented cells (NBS1 cDNA-transfected NBS cells) were irradiated with 2 Gy, and immunofluorescent staining with anti-γ-H2AX antibody showed normal formation of γ-H2AX foci in NBS cells.

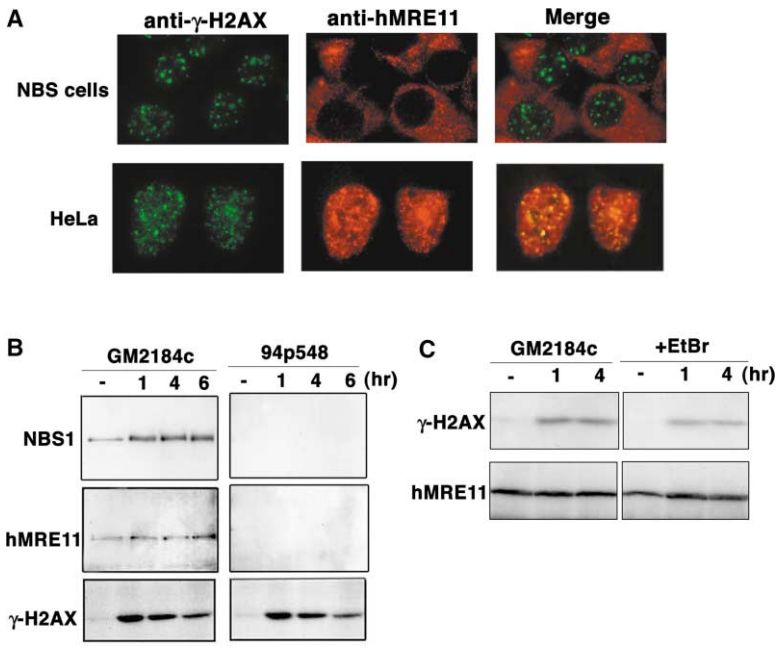

Figure 2. γ-H2AX and NBS1 Physically Interacted and Formed Foci at the Same Nuclear Sites after Irradiation

(A) HeLa cells and NBS cells (GM07166VA7) were fixed 2 hr after irradiation with 2 Gy and were double-immunofluorescent stained with anti-γ-H2AX antibody and anti-hMRE11 antibody. It is noted that both foci colocalized in HeLa cells and that hMRE11 were confined to the cytoplasm in NBS cells.

(B) Extracts from normal (GM2184c) and NBS (94p548) lymphoblastoid cells were immunoprecipitated with anti-γ-H2AX antibody at the indicated times after 10 Gy irradiation. Western blot analysis showed the presence of NBS1/hMRE11 in immuno-complex with anti-γ-H2AX antibody.

(C) Similarly, immunoprecipitates with anti-NBS1 antibody contained γ-H2AX in complex with NBS1, and this physical interaction was not affected by treatment with ethidium bromide (50 μg/ml).

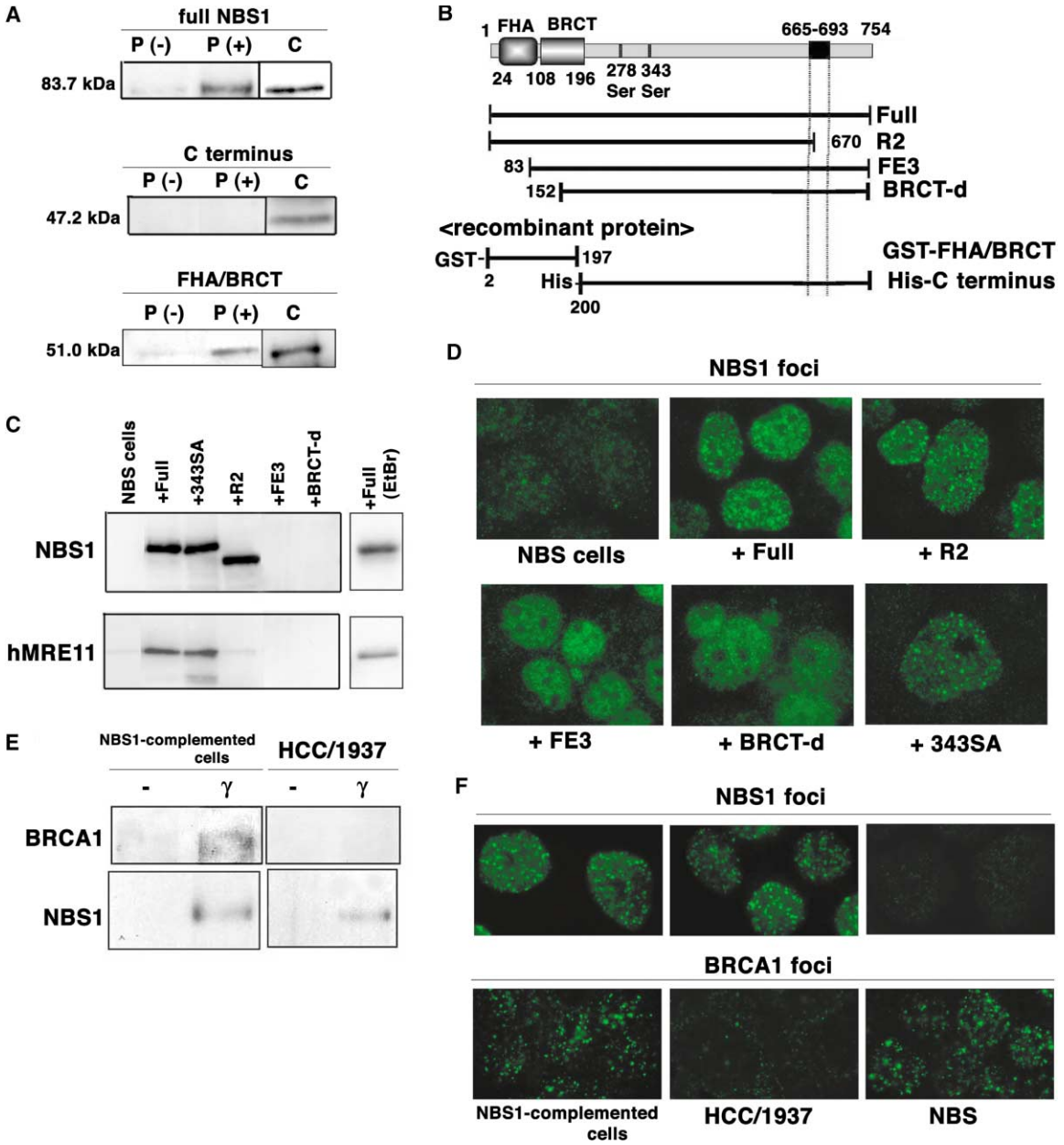

Figure 3. NBS1 Directly Bound to γ-H2AX Via the FHA/BRCT Domain Both In Vitro and in Cells

(A) A mixture of 1 μg recombinant full-length NBS1, C-terminal NBS1, or the FHA/BRCT-GST-tagged protein with 1 μg recombinant H2AX (P[−]: nonphosphorylated H2AX, P[+]: H2AX phosphorylated by ATM kinase) was immunoprecipitated with anti-H2A antibody (recognizes both H2AX and γ-H2AX), and Western blot analysis indicated that full-length NBS1 directly bound to γ-H2AX, but not to nonphosphorylated H2AX (10 ng recombinant full-length NBS1 and C-terminal NBS1 were used as controls in lane C). Similarly, immuno-complex of γ-H2AX (by anti-H2A antibody) contained FHA/BRCT-GST-tagged protein, but not recombinant C-terminal NBS1.

(B) NBS fibroblast cells were transfected with several mutant NBS1 proteins, as well as an R2 construct that is truncated in the hMRE11 binding region (653–693 aa), an FE3 construct with the FHA domain deleted (24–102 aa), a BRCT-d construct with the FHA/BRCT domain deleted (108–196 aa), and an 343SA construct with serine residue 343 substituted with an alanine [4]. Recombinant NBS1 proteins used for in vitro experiments were also shown.

(C) The extracts from deletion mutant clones, fixed 1 hr after irradiation with 10 Gy, were immunoprecipitated with anti-γ-H2AX antibody, and Western blot analysis showed the presence of NBS1 in immuno-complex of γ-H2AX in Full, S343A, and R2 clones, but not in FE3 and BRCT-d clones lacking the FHA domain or FHA/BRCT domain. Similarly, γ-H2AX was present in immuno-complex of NBS1 in Full and R2 clones.

(D) Immunofluorescent staining of the NBS1 deletion mutant clones in (C) indicated normal NBS1 foci formation in Full, 343SA, and R2 clones, but not in FE3 and BRCT-d clones.

(E) NBS1-complemeted cells and HCC/1937 cells were fixed 1 hr after irradiation with 10 Gy, and the extracts were immunoprecipitated with anti-γ-H2AX antibody. NBS1 was contained in this immuno-complex from HCC/1937 lacking active BRCA1. BRCA1 was present in this immuno-complex from NBS1-complemented cells, but not from HCC/1937 cells.

(F) NBS1-complemented cells and HCC/1937 cells in (E) were immuno-stained with anti-NBS1 antibody (upper panels) and anti-BRCA1 antibody (lower panels). HCC/1937 cells, deficient in the formation of BRCA1 foci, formed normal NBS1 foci.

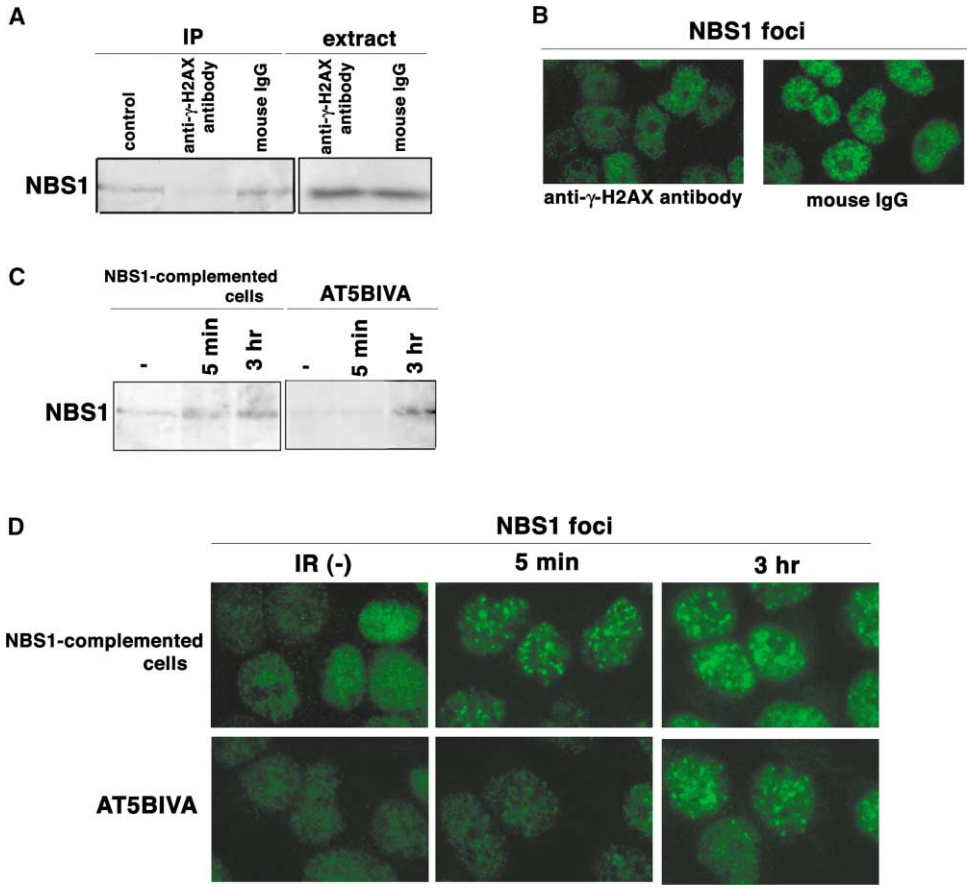

Figure 4. The Interaction of γ-H2AX and NBS1 Was Reduced by Blockage of γ-H2AX

(A) A total of 1 μg γ-H2AX mouse monoclonal antibody or mouse IgG was introduced into HeLa cells with Chariot-reagent (Active Motif) according to the manufacturer's protocol, and, 1 hr after irradiation with 5 Gy, the extracts from the cells were immunoprecipitated with anti-γ-H2AX rabbit polyclonal antibody. Western blot analysis with NBS1 antibody showed that introduction of antibody significantly reduced the amount of NBS1 in the immuno-complex with γ-H2AX.

(B) HeLa cells introduced with anti-γ-H2AX antibody or mouse IgG in (A) were immuno-stained with anti-NBS1 antibody. NBS1 foci formation was significantly reduced after the introduction of anti-γ-H2AX antibody.

(C) After irradiation with 2 Gy, extracts from NBS1-complemented cells and AT fibroblast cells (AT5BIVA) were immunoprecipitated with anti-γ-H2AX antibody. Western blot analysis showed a reduced amount of NBS1 in the immuno-complex of γ-H2AX from AT cells, when compared 5 min after irradiation.

(D) Immuno-staining of the cells in (C) showed a reduced number of NBS1 foci in 5-min postirradiated AT cells.

amino acids are required for hMRE11 binding, and the FHA/BRCT domain is indispensable for NBS1 foci formation [4]. We generated several mutants of NBS1 to determine the domain responsible for interaction with γ-H2AX (Figure 3B). Similar to wild-type NBS1 and an NBS1 (343SA) construct, the NBS1-R2 (truncation involving hMRE11 binding domain) construct was coimmunoprecipitated with anti-γ-H2AX antibody (Figure 3C). Hence, the NBS1-R2 construct binds γ-H2AX and forms foci in the absence of hMRE11 (Figure 3D). Deletion constructs of NBS1 lacking either or both of the FHA and BRCT domains were not immunoprecipitated with anti-γ-H2AX antibody (Figure 3C) and also failed to form NBS1 (Figure 3D) or hMRE11 foci (data not shown). These experiments demonstrated that both NBS1/γ-H2AX interaction in cells and NBS1 foci formation require the FHA/BRCT domain of NBS1, but that such activity is not mediated through interaction with hMRE11/hRAD50. This is supported by the observation that NBS1 bound to γ-H2AX and formed the foci in HCC/1937 cells lacking active BRCA1 (Figures 3E and 3F), since it is excluded that NBS1 binding to γ-H2AX is mediated through hMRE11/hRAD50/BRCA1 complex [7].

As described above, H2AX was rapidly phosphorylated in the absence of NBS1. We speculated that phosphorylation of H2AX was first recognized by NBS1 and was followed by formation of NBS1 foci, in which hundreds to thousands of proteins were recruited and accumulated. To investigate this, we examined the NBS1/γ-H2AX interaction and NBS1 foci formation after masking the epitope of γ-H2AX phosphoprotein; masking the epitope also might inhibit the conversion of H2AX to γ-H2AX in contiguous units of histone. When anti-γ-H2AX antibody was introduced to normal cells, immunoprecipitation of NBS1 with anti-γ-H2AX antibody and formation of NBS1 foci were strikingly inhibited, whereas both foci formation and NBS1/γ−H2AX interaction were not significantly affected by introduction of nonspecific

IgG (Figures 4A and 4B). H2AX is known to be phosphorylated in normal cells by ATM kinase shortly after irradiation; hence, ATM-mutated AT cells exhibited delayed phosphoryation of H2AX (data not shown). As a result, NBS1 binding to γ-H2AX was reduced (Figure 4C), and very few NBS1 foci appeared in irradiated AT cells 5 min after irradiation (Figure 4D), while the number of foci was restored to the normal level 3 hr later. Since both antibody-introduced cells and AT cells carry the same number of DNA double-strand breaks as do normal control cells, NBS1 recognizes γ-H2AX, rather than DNA double-strand breaks, and recruits hMRE11/hRAD50 to form foci on damaged sites.

We demonstrated here that NBS1 directly binds to histone γ-H2AX, phosphorylated after DNA damage, which is consistent with a recent report by Celeste et al., who indicated that mouse cells lacking histone H2AX were impaired in recruitment of NBS1, 53BP1, and BRCA1, but not hRAD51, to damaged DNA [8]. There are at least two classes of protein, which form foci on sites of DNA double-strand breaks; proteins containing the BRCT domain, such as NBS1, 53BP1, and BRCA1 [4, 7, 9–11], and those such as hRAD51 [12], which directly binds to DNA but has no BRCT domain. The present results indicated that NBS1, the first type, interacts with γ-H2AX, rather than damaged DNA, via the FHA/BRCT domain (Figure 3A) and accumulates as foci in the vicinity of damaged sites (Figure 3D). This is consistent with the observation that in the breast cancer, cell line HCC/1937, in which the BRCT domain is truncated, fails to form BRCA1 foci [7], and cells lacking the 5th BRCT domain of TOPBP1 showed no TOPBP1 foci after irradiation [13]. On the other hand, NBS1 can bind to γ-H2AX in the absence of the interaction with hMRE11/hRad50 (Figure 3C) or BRCA1 (Figure 3E) in an initial response to ionizing radiation, although NBS1 foci formation mediated through hRAD50 binding to BRCA1 has been suggested in cells 8 hr postirradiation [7]. Thus, BRCT domain-containing proteins such as NBS1, BRCA1, and 53BP1 appear to interact with histone independently, since BRCA1 and 53BP1 foci formation was not affected by the absence of NBS1 (Figure 3F) [10] and (vice versa) NBS1 foci were formed without BRCA1 foci (Figure 3F) [14]. Such foci formation, known as the first step in a two-step binding mechanism for damage recognition [15], could facilitate DNA repair and cell cycle monitoring; consequently, abrogated function of FHA/BRCT domains may lead to genomic instability and carcinogenesis, clinical phenotypes displayed by patients with NBS and familial breast cancer.

Experimental Procedures

Cell Line

NBS fibroblast cell line GM07166VA7 was established by SV40 transformation of GM07166 cells, which were provided from NIGMS Cell Repository. SV40-transformed AT fibroblast cell line AT5BIVA, AT28 cells, and NBS lymphoblastoid cell line 94p548 were kindly supplied by Dr. A. Lehmann, Dr. M. Oshimura, and Dr. K. Sperling, respectively. Normal lymphoblastoid cell line GM2184c and mammal gland adenocarcinoma HCC/1937 (5382insC BRCA1) were obtained from NIGMS Cell Repository and the American Type Culture Collection.

Preparation of Antibodies and Recombinant Proteins

Anti-γ-H2AX antibodies (rabbit and mouse) were prepared from a synthetic peptide consisting of the last nine amino acids of H2AX phosphorylated at serine 139 [5]. Other antibodies used for Western blot and immunoprecipitation were as follows: anti-NBS1 antibody [4], anti-hMRE11 antibody (Novus Biologicals), anti-H2A antibody (Santa Cruz), anti-GST antibody (Amersham Bioscience), and anti-His antibody (QIAGEN). The expression vectors of His-tagged NBS1 were constructed by the insertion of full-length NBS1 cDNA or truncated NBS1 cDNA (deleted in 1–199 aa) into pQE32 vector (QIAGEN) and transformation in *E. coli* M15 (QIAGEN). The expression vector of the GST-tagged FHA/BRCT domain of NBS1 was prepared by the insertion of human truncated NBS1 cDNA (2–197 aa) into pGEX-2T vector (Amersham Biosciences) and transformation in *E. coli* JM109 (TOYOBO).

Immunofluorescent Staining

Cells grown on a glass slide were fixed with cold methanol for 15 min, rinsed with cold acetone several times, and then air dried. The slides were stained as described previously [4]. Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) or Alexa-546-conjugated anti-mouse IgG (Molecular Probes) were used for visualization of foci with anti-γ-H2AX antibody, anti-NBS1 antibody, or anti-hMRE11 antibody by an Olympus fluorescent microscope.

Supplementary Material

Supplementary Material including figures showing the time course of γ-H2AX foci disappearance after irradiation that explains nonoverlapping foci of hMRE11 with γ-H2AX and purification of recombinant NBS1 and the binding ability to hMRE11 is available at http://images.cellpress.com/supmat/supmatin.htm.

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